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# Recognition of tRNA by Isoleucyl-tRNA Synthetase

## Effect of Substrates on the Dynamics of tRNA–Enzyme Interaction

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An interaction between the isoleucine catalytic site and the tRNA recognition site of isoleucyl-tRNA synthetase (*Escherichia coli*) was detected using an assay for the binding of tRNA to enzyme. The effect of isoleucine is to increase the rate at which tRNA enters and leaves its binding site by sixfold without, therefore, a large effect on the equilibrium constant for tRNA binding. This effect requires neither transfer of isoleucine to tRNA nor activation of isoleucine as isoleucyl-AMP; the binding of isoleucine alone suffices. It appears from these data that release of aminoacyl-tRNA is the rate-limiting step in the acylation, or transfer, reaction. The apparent maximum rate constant for association of tRNA and enzyme is relatively large, $6 \times 10^6$ M$^{-1}$ sec$^{-1}$ (17°C).

## 1. Introduction

To study the specific affinity of aminoacyl-tRNA synthetases for their cognate tRNA's, we have developed an assay that measures this binding directly; that is, the measurement is independent of the esterification of amino acid to tRNA (Yarus & Berg, 1967).

Early in the study of the recognition of tRNA by isoleucyl-tRNA synthetase, we noted that tRNA, once bound, is released from the enzyme very slowly, e.g. 10 to 15 minutes are required for most of the IRS(tRNA$^{Ile}$)† complex to dissociate. This was puzzling because such a slow rate of dissociation could not account for the observed rate of the acylation reaction; on simple assumptions, the turnover rate could not be

† Abbreviations and notation: Standard abbreviations have been used throughout. tRNA$^{Ile(2)}$ refers to peak 2 of the tRNA$^{Ile}$ in our chromatographic system.

The various non-covalent associations of which isoleucyl-tRNA synthetase is capable are indicated, by the use of brackets enclosing the ligands: for example, IRS(Ile-tRNA) or IRS(Ile-AMP) symbolizes the isoleucyl-tRNA synthetase molecule which has respectively bound an Ile-tRNA or an Ile-AMP. IRS(Ile-tRNA)(Ile-AMP) symbolizes the ternary complex of enzyme with both Ile-tRNA and Ile-AMP.

tRNApCpCpA is an unmodified tRNA molecule, in which the 3′ nucleotides are specifically indicated, and tRNApCpC and tRNApC are the molecules with a pA or pCpA, respectively, removed. HMV-tRNA is α-hydroxy-β-methyl valeryl-tRNA.

faster than the rate at which acylated tRNA is released by the enzyme. A search for the difference between the conditions used to measure binding and those used to measure the esterification of isoleucine to $tRNA^{Ile}$ revealed that the important factor is the presence of L-isoleucine. Saturation of the enzyme with L-isoleucine enhances the rate of release of tRNA, and the maximum rate of release is similar to the maximum rate of the acylation reaction.

## 2. Materials and Methods

### (a) *Enzymes*

Isoleucyl tRNA synthetase is the pure, homogeneous protein prepared and characterized by Baldwin & Berg (1966). Snake venom phosphodiesterase is the Worthington (Freehold, N.J.) preparation, VPA, purified further by the method of Keller (1964). Transfer RNA CCA-pyrophosphorylase [ATP(CTP) : RNA nucleotidyl transferase] is a 30-fold purified preparation from *Escherichia coli* B, made by the method of Hurwitz & Furth (1966). It does not degrade tRNA as judged by assay of isoleucine-acceptor activity following incubation of tRNA with the enzyme preparation.

### (b) *tRNA*

In most experiments, tRNA was a highly purified preparation of $tRNA^{Ile}$ (*E. coli* B). Briefly, tRNA prepared by the method of Zubay (1962) or bought from Schwarz Bioresearch as *E. coli* B tRNA (lot no. 1110-40) was fractionated according to a modification of the procedure of Gordon Tener (personal communication, and quoted in Reeves *et al.*, 1968). The tRNA was acylated with purified IRS, and the Ile-amino group was phenoxyacetylated using *N*-phenoxyacetyl-succinimide. The completeness of the phenoxyacetylation was checked by measuring the fraction of Ile-tRNA which could not be enzymically deacylated in the presence of AMP and $PP_i$: the derivative is not deacylated. The addition of the hydrophobic phenoxyacetyl residue enables the aminoacylated tRNA to be separated from unacylated tRNA's which are unreactive to the *N*-phenoxyacetyl-succinimide. About 10,000 $A_{260}$ units of the charged, phenoxyacetylated tRNA in 0·3 M-NaCl–0·01 M-$MgCl_2$ was applied to a 2 cm × 100 cm column of benzoylated DEAE (Gillam *et al.*, 1967) at 4°C. After collecting the runoff at 120 ml./hr, elution with 1·0 M-NaCl–0·01 M-$MgCl_2$ was begun; this eluted most of the unacylated tRNA's. The remainder was largely removed using gradient elution, which began when the absorbance in the 1·0 M-NaCl eluant had decreased to 1·0 (254 mμ). A gradient of 400 ml. each of 1·0 M-NaCl–0·01 M-$MgCl_2$ to 2·0 M-NaCl–0·01 M-$MgCl_2$ containing 25% ethanol eluted the remaining unacylated tRNA's as a single peak and then, superposed on the tail end of this peak was a small but definite peak containing all the *N*-phenoxyacetyl-Ile-tRNA. This was concentrated by dialysis *versus* Sephadex G200 (Pharmacia, Piscataway, N.J.) and then deacylated by treatment for 20 min at 25°C with 0·05 M-$Na_2CO_3$, pH 10. At this point, the Ile-tRNA is about 40% pure, based on an expected level of charging for pure $tRNA^{Ile}$ of 1·48 mμmoles of isoleucine per $A_{260}$ unit, measured in 0·01 N-NaOH. The tRNA is further purified and the isoacceptors separated on a column of benzoylated DEAE (0·8 cm × 100 cm), run at 12 ml./hr with a linear gradient between 0·5 M-NaCl–0·01 M-$MgCl_2$ and 1·25 M-NaCl–0·01 M-$MgCl_2$, total volume, 400 ml. Separated peaks are pooled and concentrated by dialysis *versus* Sephadex. There are generally four distinguishable regions: a leading diffuse region ($tRNA^{Ile(1)}$), two major peaks ($tRNA^{Ile(2)}$ and $tRNA^{Ile(3)}$ and a trailing region ($tRNA^{Ile(4)}$). In the major peaks, the absorbance and acceptor activity curves coincide and the specific activity is about 1·0 mμmole of isoleucine/$A_{260}$. This represents a 50-fold purification of the acceptor in Schwarz *E. coli* B tRNA and a 25-fold purification of $tRNA^{Ile}$ in the tRNA prepared according to Zubay (1962).

### (c) *$^{14}C$-labeled α-hydroxy-β-methylvaleryl-$tRNA^{Ile}$*

This derivative is useful when specific labeling of $tRNA^{Ile}$ chains is needed, and insensitivity to enzymic or non-enzymic deacylation is desired. It was prepared by nitrous acid treatment (Herve & Chapeville, 1965) of the isoleucine moiety of Ile-tRNA; this

removes the free amino group and replaces it with an hydroxyl. [$^{14}$C]Ile-tRNA$^{Ile(3)}$ is combined with 0·5 vol. saturated $NaNO_2$ solution, and 1/200 vol. glacial acetic acid and incubated for 15 min at 25°C. The HMV-tRNA$^{Ile(3)}$ is then precipitated 4 times in succession by addition of 0·1 vol. of 20% potassium acetate, pH 5·5, and 2 vol. of cold 100% ethanol. The final precipitate was dried in a stream of air; the resulting $^{14}$C-labeled HMV-tRNA$^{Ile(3)}$ retains all the $^{14}$C of the original Ile-tRNA$^{Ile(3)}$, but is insensitive to enzymic deacylation by IRS in the presence of AMP and $PP_i$, under conditions which deacylate $> 95\%$ of a similar amount of Ile-tRNA$^{Ile(3)}$. In addition, the association constant of $^{14}$C-labeled HMV-tRNA$^{Ile(3)}$ and IRS measured by the binding to nitrocellulose filters (Yarus & Berg, 1967) is indistinguishable from that of the starting material, Ile-tRNA$^{Ile(3)}$. Thus, the brief nitrous acid treatment, which would not be expected to produce a significant deamination of ribonucleotides (Carbon, 1965), has not affected the recognizability of the tRNA.

### (d) *Periodate-oxidized tRNA; tRNA(CHO)$_2$*

Typically, 0·4 $\mu$mole of $NaIO_4$ was reacted with 2·6 $A_{260}$ units of tRNA$^{Ile(3)}$ in a total volume of 0·25 ml. After 60 min at 17°C in the dark, 4 $\mu$moles of ethylene glycol was added to destroy residual $IO_4^-$. The tRNA was precipitated with 0·1 vol. of 5 M-NaCl and 2 vol. of cold ethanol, dried with ethanol–ether (1 : 1, v/v) and then in a stream of dry air; this procedure was repeated 3 times. The resulting tRNA$^{Ile(3)}$(CHO)$_2$ had no detectable acceptor activity ($< 0{\cdot}2\%$ that of the starting material) and still competed as well as the starting material, tRNA$^{Ile(3)}$, with Ile-tRNA for binding to the enzyme (Yarus & Berg, 1967). Further, Ile-tRNA after treatment in this way with $IO_4^-$, is unaffected; it is deacylated in the presence of IRS, AMP and $PP_i$ at the same rate as untreated Ile-tRNA.

### (e) *tRNApCp$^{14}$C-C*

The 3′-terminal pCpA sequence of tRNA$^{Ile(3)}$ was removed by snake venom phosphodiesterase at low temperature using a procedure described by Zubay & Takanami (1964). For these experiments, 3·08 $A_{260}$ units of tRNA$^{Ile(3)}$ were treated for 10·5 hr at 17°C in 1 ml. of 0·01 M-Tris–HCl, pH 7·5, 0·01 M-$MgCl_2$ with an amount of purified snake venom phosphodiesterase sufficient to release 0·027 $\mu$mole of *p*-nitrophenol/min at 30°C from *p*-nitrophenyl-5-thymidylyl phosphate, at pH 9·0 in 0·06 M-Tris–HCl, plus 0·05 M-$MgCl_2$.

After incubation, the reaction was treated with an equal volume of water-equilibrated phenol and the putative tRNApC recovered from the aqueous phase by multiple precipitation from 0·5 M-NaCl and ethanol as described above. The product has no detectable isoleucine-acceptor activity ($< 0{\cdot}3\%$ that of tRNA$^{Ile(3)}$), but still competes well in the binding to IRS (association constant = 0·75 that of tRNA$^{Ile(3)}$). When incubated with tRNA CCA-pyrophosphorylase and [$^{14}$C]CTP (149 $\mu$c/$\mu$mole, Schwarz Bioresearch), the putative tRNApC accepts a maximum of 1·53 m$\mu$moles [$^{14}$C]CMP/$A_{260}$ of the RNA. This is 1·03 moles of CMP/mole of tRNA, and argues strongly, because of the specificity of CCA-pyrophosphorylase (Preiss, Dieckmann & Berg, 1961), that the structure of the phosphodiesterase product is tRNA$^{Ile(3)}$pC. Removal of more than the sequence of pCpA would be expected to lead, first, to the acceptance of $> 1$ mole CMP/mole tRNA, and then, if more than the sequence pCpCpA is removed, to loss of CMP acceptor activity entirely.

To determine the fraction of the $^{14}$C-labeled tRNA chains which are tRNA$^{Ile}$, the tRNApCp$^{14}$C-C was incubated with IRS and filtered through a Schleicher & Schuell B6 filter under binding conditions (Yarus & Berg, 1967); the tRNA in the filtrate is reincubated with enzyme and re-filtered. In this way, all tRNA which is recognizable by IRS is bound to filters; $^{14}$C not retained by the filters is not associated with tRNA$^{Ile}$. When this test is applied to [$^{14}$C]Ile-tRNA, $> 98\%$ of the radioactivity is bound to filters: with the preparation of tRNA$^{Ile(3)}$pCp$^{14}$C-C mentioned above, 57% of the $^{14}$C was bound. Since the starting tRNA$^{Ile(3)}$ was about 61% tRNA$^{Ile}$ (9·0 m$\mu$moles of Ile/10 $A_{260}$), we may conclude that almost all tRNA$^{Ile(3)}$ chains have been converted to tRNA$^{Ile(3)}$pCp$^{14}$C-C, and are still recognizable to IRS.

(f) *Assays*

ATP–$^{32}PP_i$ exchange is measured by terminating the reaction with 0·5 ml. of 15% perchloric acid containing 0·4 M-sodium pyrophosphate, absorbing ATP to 15 mg of activated charcoal and collecting, washing, and counting the charcoal on a Whatman glass fiber disk (GF/C, 24 mm). The acylation reaction has been described (Yarus & Berg, 1967).

The quantity of $tRNA^{Ile}$ is always determined by conversion to [$^{14}$C]Ile-tRNA and the amount of active IRS by formation and isolation of IRS(Ile-AMP) (Norris & Berg, 1964).

The tRNA binding to IRS was as described previously (Yarus & Berg, 1967). The sole modification is the treatment of the nitrocellulose filters used to adsorb IRS(tRNA) complexes: they are soaked 24 hr or more in wash fluid before use, as this was found to lower the background of counts retained on the filter in the absence of enzyme. The reaction mixtures contain 0·044 M-$KH_2PO_4$, 0·006 M-$K_2HPO_4$, 0·01 M-$MgCl_2$, 0·01 M-2-mercaptoethanol and 10 $\mu$g of BSA; the final pH is 5·5. The detection of IRS(tRNA) complex depends on the retention of the complex by nitrocellulose (Schleicher & Schuell B6, 24 mm) filters, under conditions in which tRNA is completely filterable (Yarus & Berg, 1967). A $\mu\mu$mole of [$^{14}$C]Ile-tRNA bound is equivalent to 440 cts/min, under our conditions.

## 3. Results

### (a) *Observation and measurement of the effect of substrates on dissociation of IRS($tRNA^{Ile}$)*

The dissociation of bound $tRNA^{Ile}$ from IRS to reach a new steady state is readily seen when the IRS([$^{14}$C]Ile-tRNA) complex is quickly diluted (Fig. 1). In the presence of ATP and isoleucine or isoleucine alone, the adjustment is over quickly while, in the absence of substrates, the dissociation is slow and has not reached the new level even after 12 minutes at 17°C. This disappearance of bound $^{14}$C in the presence of isoleucine or ATP and isoleucine is not due to removal of [$^{14}$C]isoleucine from tRNA since all of the $^{14}$C is still precipitable as [$^{14}$C]Ile-tRNA at the end of the experiment.

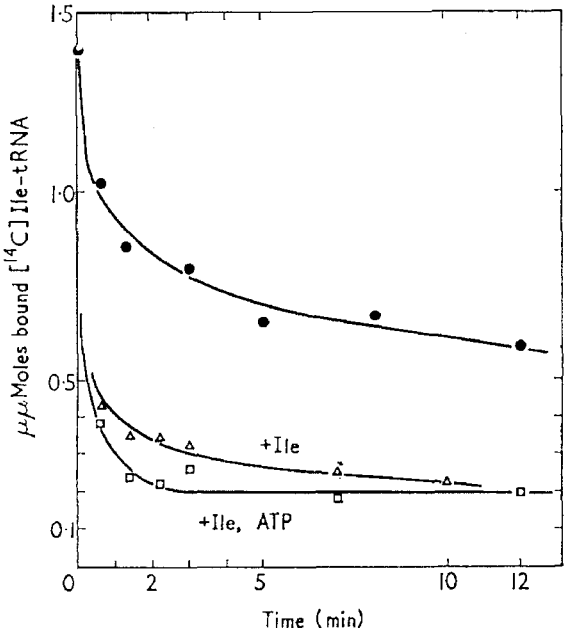


FIG. 1. Release of bound Ile-tRNA from the IRS(Ile-tRNA) complex after dilution.

15·1 $\mu\mu$moles of [$^{14}$C]Ile-$tRNA^{(2)}$ and 15·1 $\mu\mu$moles of IRS in 0·040 ml. at 17°C were diluted to 40 ml. in binding assay solution with no other additions (—●—●—), the same reaction with the addition of $5\times10^{-4}$ M-isoleucine was diluted in the presence of isoleucine (—△—△—), or a similar reaction with the addition of $5\times10^{-4}$ M-ATP and $5\times10^{-4}$ M-isoleucine was diluted in the presence of ATP and isoleucine (—□—□—). 6-ml. samples were filtered at intervals.

The required filtration of large volumes makes the dilution experiment cumbersome for kinetic studies. A more convenient way to measure the rate of dissociation of IRS(tRNA$^{Ile}$),

$$IRS(tRNA) \rightleftarrows IRS + tRNA \quad (1)$$

is by tRNA *exchange*. This procedure is also performed under conditions more analogous to those encountered during the normal function of IRS, since the exchange of tRNA's is necessarily a part of the over-all acylation reaction. In this method, the complex is first formed with [$^{14}$C]Ile-tRNA and then unlabeled tRNA$^{Ile(2)}$ is added in a small volume and the replacement of the [$^{14}$C]Ile-tRNA by unlabeled tRNA$^{Ile(2)}$ is followed (Fig. 2). Since (as shown below) the rate of dissociation is independent of

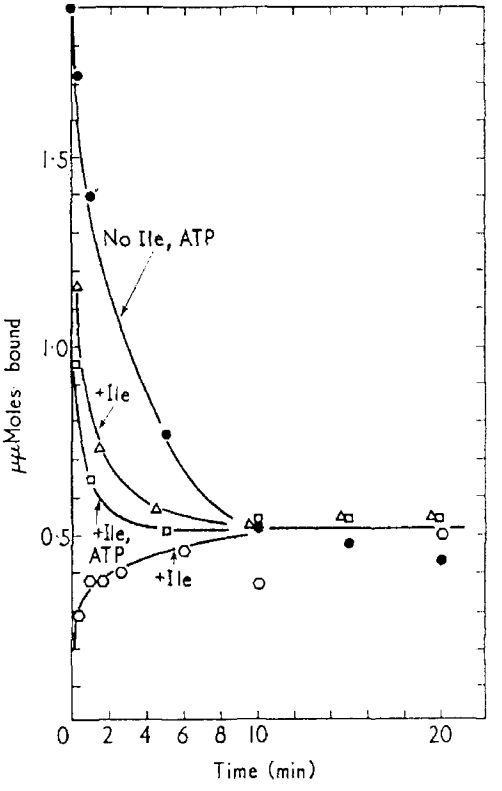


FIG. 2. Interchange of IRS-bound [$^{14}$C]Ile-tRNA with free tRNA$^{Ile}$ or the reverse.
46 $\mu\mu$moles of [$^{14}$C]Ile-tRNA$^{(2)}$ and 23 $\mu\mu$moles of IRS were combined in 0·40 ml. at 17°C, and 115 $\mu\mu$moles of tRNA$^{Ile(2)}$ added. At intervals afterward, 0·050-ml. samples were filtered (—●—●—). Where indicated, the above reactants were supplemented with $5 \times 10^{-4}$ M-isoleucine (—△—△—) or $5 \times 10^{-4}$ M-isoleucine and $6 \times 10^{-4}$ M-ATP (—□—□—). In another experiment, performed in the presence of isoleucine only, the order of addition of tRNA's was reversed (—○—○—).

the concentration of the tRNA$^{Ile(2)}$, the rate-limiting step in the exchange is the dissociation of the first complex, the IRS([$^{14}$C]Ile-tRNA); therefore, the rate of approach to the new equilibrium position measures the rate of dissociation. As in Figure 1, the rate of dissociation is speeded by the presence of the small-molecule substrates of the enzyme, ATP and isoleucine, or by isoleucine alone. The exchange can also be followed by reversing the order of tRNA addition, so that, to reach the final state, the tRNA$^{Ile(2)}$ must dissociate from the enzyme and be replaced by [$^{14}$C]Ile-tRNA (Fig. 2). The convergence of all curves to the same level confirms that the association of tRNA$^{Ile}$ with IRS is a true equilibrium whose final position is

independent of the order of addition of reactants. The effectiveness of isoleucine without other additions suggests that an isoleucine binding site is coupled to the recognition site; indeed, in an experiment in which IRS(tRNA$^{Ile(4)}$) was exchanged with $^{14}$C-labeled HMV-tRNA$^{Ile(4)}$, neither ATP alone (0·5 to $2·5\times10^{-3}$ M) nor ADP ($1·2\times10^{-3}$ M) nor AMP ($0·87\times10^{-3}$ M) has any detectable effect on the rate of exchange (Table 3).

To show more clearly the effect of substrates, the kinetics at early times after mixing was examined more closely (Fig. 3(a)). As observed in a similar experiment

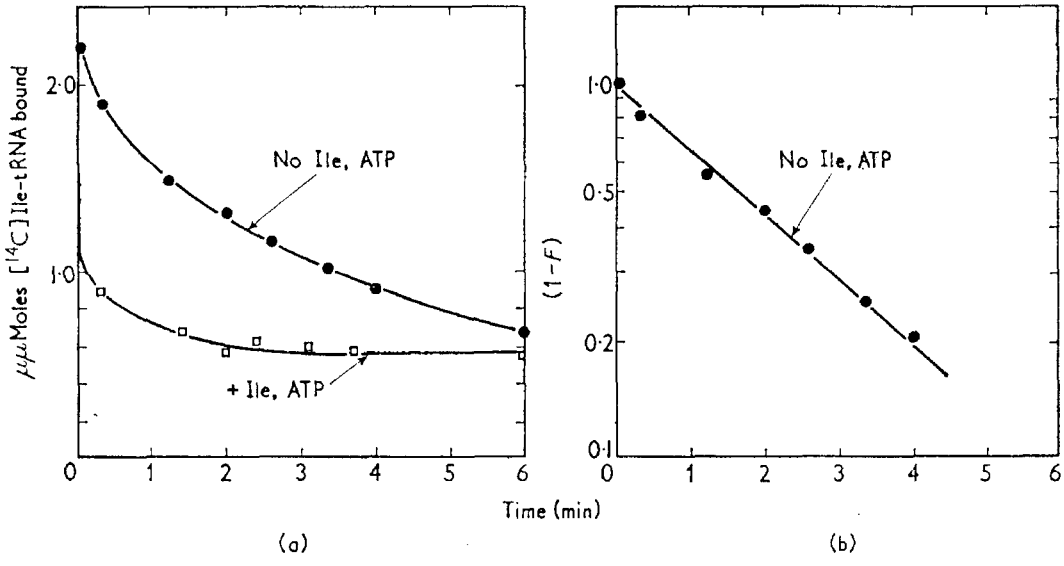


FIG. 3. (a) Effect of substrates on the rate of exchange of bound [$^{14}$C]Ile-tRNA with free tRNA$^{Ile}$. The experiment was carried out in the same way as that described for Fig. 2.

(b) The data of the rate of exchange in the absence of substrates is replotted. $F$ is the fraction of total exchange which has occurred at the particular times. See text and the legend to Table 1 for additional explanation of the plot.

presented in Figure 2, the rate of tRNA exchange in the presence of substrates is strikingly greater. In Figure 3(b), the data of the exchange in the absence of ATP and isoleucine are plotted as $\log_{10}(1 - F)$ *versus* time; that is, the logarithm of 1 minus the fraction of the final exchange which has already taken place at the time $t$ (see the legend to Table 1). Since a straight line is obtained, we conclude that the system is homogeneous and has only one rate of exchange; or, put another way, that all tRNA's and their binding sites are equivalent (Meyers & Priestwood, 1951).

This justifies assignment of a single rate constant, as is done in Table 1. Rate constants are calculated assuming that the rate of exchange is determined by the first-order dissociation of the IRS(tRNA) complex. The agreement of the calculated first-order rate constants over a fivefold range in the amount of added tRNA$^{Ile(2)}$ implies that the rate of exchange is indeed determined in some process independent of the concentration of the second tRNA, presumably the fission of the enzyme–tRNA pair. This is in good accord with our expectation; dissociation is obviously slow (Fig. 1), and association fast (Yarus & Berg, 1967); we expect, therefore, that dissociation should limit the rate of exchange. The observation of a single rate constant, independent of total tRNA concentration, implies that an entering tRNA does not *provoke* an exchange with a bound tRNA; rather, replacement must wait on the release of the first tRNA by a process which depends only on the stability of

TABLE 1

*Rate constant for the dissociation of IRS(Ile-tRNA$^{Ile(2)}$) at 17°C*

| Concentration $tRNA^{Ile(2)}$ added (M) | Other additions | $k_d$ ($sec^{-1}$) | $k_d$, average ($sec^{-1}$) |
|---|---|---|---|
| $5{\cdot}4\times10^{-8}$ | none | $7{\cdot}0\times10^{-3}$ | |
| $1{\cdot}1\times10^{-7}$ | none | $8{\cdot}2\times10^{-3}$ | $7{\cdot}5\times10^{-3}$ |
| $2{\cdot}7\times10^{-7}$ | none | $7{\cdot}3\times10^{-3}$ | |
| $5{\cdot}4\times10^{-8}$ | ATP + Ile | $4{\cdot}0\times10^{-2}$ | |
| $1{\cdot}1\times10^{-7}$ | ATP + Ile | $4{\cdot}5\times10^{-2}$ | $4{\cdot}3\times10^{-2}$ |
| $2{\cdot}7\times10^{-7}$ | ATP + Ile | $4{\cdot}5\times10^{-2}$ | |

Reactions contained, in 0·10 ml., $5{\cdot}8\times10^{-8}$ M-IRS, $5{\cdot}8\times10^{-8}$ M-[$^{14}$C]Ile-$tRNA^{Ile(2)}$, $1{\cdot}9\times10^{-3}$ M-ATP and $1{\cdot}0\times10^{-3}$ M-isoleucine where indicated. To begin exchange, the indicated amount of $tRNA^{Ile(2)}$ was added in a few microliters. Exchange in the presence of substrates is rapid, and allows time for only one point which is a reliable distance from the equilibrium position. The rates with substrates shown in the Table were calculated from a single assay at 0·20 min, which is at the limit of manual manipulation. At this point about half the total exchange had occurred, so that the rates are based on a reliable difference between the measured and the final levels of tRNA bound. The analysis is based on the equation:

$$\log_{10}(1-F) = \frac{1}{2{\cdot}303}\,\frac{[\text{free tRNA}]+[\text{bound tRNA}]}{[\text{free tRNA}]\quad[\text{bound tRNA}]}\,Rt$$

(Meyers & Priestwood, 1951) where $F$ is the fraction of the final exchange which has taken place at time $t$, the brackets indicate concentrations of the indicated reactants and $R$ is the rate of exchange. If the dissociation of IRS(Ile-tRNA) determines $R$, then $R = k_d$ [IRS(Ile-tRNA)] in which $k_d$ is a first-order rate constant and, therefore,

$$\log_{10}(1-F) = \frac{1}{2{\cdot}303}\,\frac{[\text{free tRNA}]+[\text{bound tRNA}]}{[\text{free tRNA}]}\,k_d t.$$

A semi-logarithmic plot of $(1-F)$ *versus* $t$, as in Fig. 3(b), yields $k_d$. Under these conditions the enzyme is always saturated with $tRNA^{Ile}$, so that IRS(Ile-tRNA) = $5{\cdot}8\times10^{-8}$ M.

IRS(tRNA). Binding of isoleucine to the enzyme increases the rate of release of tRNA from the combining site about sixfold (Table 1). This effect occurs similarly with all subspecies of $tRNA^{Ile}$; it can be demonstrated even with unpurified preparations (Yarus & Berg, unpublished data).

Since isoleucine alone stimulates release of bound tRNA, it clearly requires neither activation of isoleucine to Ile-AMP nor esterification of isoleucine to the tRNA being released. This latter fact is doubly clear in Figures 1 and 2, since the leaving tRNA's already have the 3′-terminus esterified. The stimulation of exchange may even be demonstrated between tRNA's neither of which can be acylated; by first reacting the enzyme with $tRNA(CHO)_2$, the subsequent association of [$^{14}$C]Ile-tRNA with the enzyme can be followed. This observation shows, as do the data in Figure 2 and Table 3, that the isoleucine-stimulated release of bound tRNA also occurs with unacylated tRNA's. It therefore does not depend on interaction of the isoleucine residue in Ile-tRNA with isoleucine bound to the enzyme.

### (b) *Identification of the isoleucine effector site*

Because the isoleucine-stimulated dissociation of IRS(tRNA) occurs in the absence of ATP, one may wonder if the catalytic site (that is, the site that binds the isoleucine moiety of Ile-AMP) is involved at all. Conceivably, there might be distinct effector

sites on IRS which bind isoleucine and thereby control the configuration of the tRNA site. Perhaps, alternatively, isoleucine affects $tRNA^{Ile}$ directly. The following experiments show, however, that binding of isoleucine to the enzyme's catalytic site alone is sufficient to change the tRNA site.

Of the standard set of 20 amino acids, only isoleucine or valine plus ATP influenced the rate of the tRNA exchange reaction (Table 2). The stimulation by valine and ATP

TABLE 2

*Effect of substrates and different amino acids on exchange of bound and free tRNA*

| Additions | Stimulation of exchange ($\Delta$ in $\mu\mu$moles) |
|---|---|
| ATP + L-isoleucine | 1·6 |
| ATP + L-valine | 1·0 |
| L-isoleucine | 1·1 |
| L-valine | 0·0 |
| $PP_i$ | 0·1 |
| L-aspartate, L-leucine, L-methionine | 0·0 |
| L-histidine, L-phenylalanine, glycine | 0·0 |
| L-proline, L-glutamine, L-lysine | 0·1 |
| L-serine, L-arginine, L-asparagine | 0·0 |
| L-tyrosine, L-alanine, L-glutamine | 0·1 |
| L-tryptophan, L-threonine, L-cysteine | 0·0 |

5·7 $\mu\mu$moles of IRS were combined with 11·5 $\mu\mu$moles of [$^{14}$C]Ile-$tRNA^{Ile(2)}$ in 0·10 ml. After 1 min at 17°C, 23 $\mu\mu$moles of $tRNA^{Ile(2)}$ were added to begin exchange and a sample was assayed for the amount of IRS([$^{14}$C]Ile-tRNA) 0·5 min later. All amino acids were present, when indicated, at $0{\cdot}5 \times 10^{-3}$ M, ATP at $0{\cdot}73 \times 10^{-3}$ M, and $PP_i$ at $1{\cdot}0 \times 10^{-3}$ M. The column headed "Stimulation of exchange" is the amount of exchange observed in excess of that seen in the absence of substrates. 3·0 $\mu\mu$moles of bound [$^{14}$C]Ile-tRNA were detected after 0·5 min in the sample with no additions. It should be noted that "Stimulation of exchange" is related to, but may not be compared directly with, the rate constant for exchange (Table 1).

is probably due to the fact that IRS can convert valine to the IRS(Val-AMP) complex (Berg, Bergmann, Ofengand & Dieckmann, 1961). The specificity of the effector site, then, is the same as that known for the catalytic site.

In Table 3 are data which show that neither AMP, ADP, nor ATP alone has a stimulatory effect on tRNA exchange. Note that it is necessary to use HMV-tRNA (see Materials and Methods) in these experiments to avoid the deacylation of Ile-tRNA that would occur by partial reversal of the acylation reaction. The affinity of the effector site for isoleucine is markedly enhanced by the presence of ATP. When ATP is present, the concentration of isoleucine that is needed to stimulate tRNA exchange is

TABLE 3

*Effect of nucleotides on the exchange of tRNA's*

| Additions | Concentration (M) | Stimulation of exchange ($\Delta$ in $\mu\mu$moles) |
|---|---|---|
| L-Isoleucine | $0{\cdot}5\times10^{-3}$ | 0·70 |
| ADP | $1{\cdot}2\times10^{-3}$ | 0·0 |
| AMP | $0{\cdot}87\times10^{-3}$ | − 0·2 |
| ATP | $0{\cdot}50\times10^{-3}$ | − 0·1 |
| ATP | $1{\cdot}0\times10^{-3}$ | 0·0 |
| ATP | $2{\cdot}5\times10^{-3}$ | 0·0 |

11·5 $\mu\mu$moles of IRS were combined with 27 $\mu\mu$moles of $tRNA^{Ile(4)}$ in 0·10 ml. at 17°C. After 0·5 min, 4·8 $\mu\mu$moles of $^{14}$C-labeled HMV-$tRNA^{Ile(4)}$ were added to begin exchange and 0·5 min later the reaction was assayed for IRS($^{14}$C-labeled HMV-$tRNA^{Ile(4)}$). In this case, the tRNA being assayed is exchanging *onto* the enzyme. The column headed stimulation of exchange is the observed binding minus that seen in the control, which contained no additions, and in which 0·67 $\mu\mu$mole of $^{14}$C-labeled HMV-tRNA was bound. It should be noted that stimulation of exchange is related to, but may not be compared directly with, the rate constant for exchange (Table 1).

over 100-fold less than in the absence of ATP (Fig. 4). These data are consistent with enhanced binding at the effector site by formation of isoleucyl-adenylate. Because only one Ile-AMP is bound per enzyme particle (Norris & Berg, 1964), it seems most reasonable that the effector and catalytic sites are the same. Furthermore, the concentrations of isoleucine that stimulate acylation and tRNA exchange (in the presence

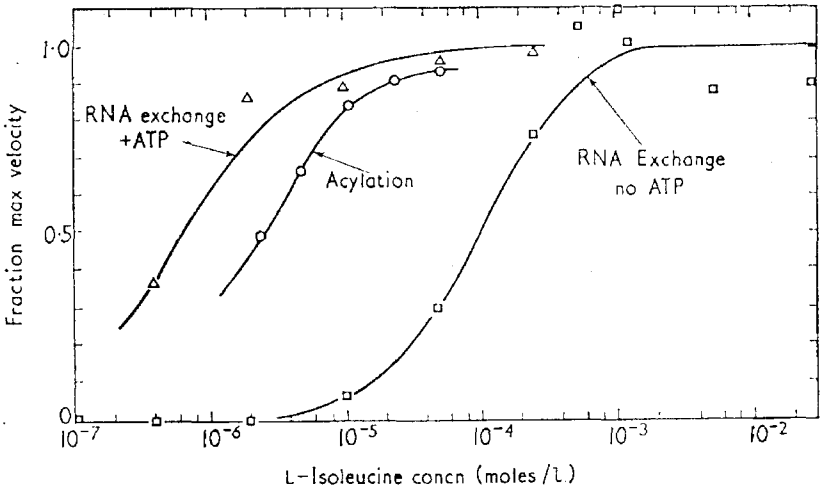


FIG. 4. Dependence of tRNA exchange and of Ile-tRNA formation on isoleucine concentration. Ile-tRNA synthesis (—○—○—) was carried out at 17°C under the same conditions used for the binding assay with the addition of $1{\cdot}9\times10^{-3}$ M-ATP, 115 $\mu\mu$moles of $tRNA^{Ile(2)}$, 1·5 $\mu\mu$moles of IRS and various concentrations of isoleucine. Stimulation of exchange was measured using 11·2 $\mu\mu$moles of $^{14}$C-labeled HMV-$tRNA^{Ile(4)}$, 11·5 $\mu\mu$moles of IRS, $1{\cdot}9\times10^{-3}$ M-ATP, and various concentrations of isoleucine (—△—△—); 55 $\mu\mu$moles of $tRNA^{Ile(3)}$ is used as the competitor tRNA in a total reaction volume of 0·20 ml. IRS($^{14}$C-labeled HMV-tRNA) was measured at 0·25 min. The amount exchanged in excess of the control normalized by the maximum exchange observed is plotted on the ordinate. Exchange of tRNA's in the presence of isoleucine alone is measured as above, except for the deletion of ATP, and use of 2·5 $\mu\mu$moles of [$^{14}$C]Ile-$tRNA^{Ile(2)}$ as the tRNA added first. Variability in measured tRNA exchange at high isoleucine concentrations illustrates the difficulty of measuring these fast exchanges.

of ATP) are similar (Fig. 4), and this is an additional argument that they employ the same site.

The most definitive evidence, however, is that when isoleucine is inserted specifically into the catalytic site and no other, the exchange of tRNA's is stimulated. This experiment is described in Figure 5, and uses the partial reverse of the aminoacylation

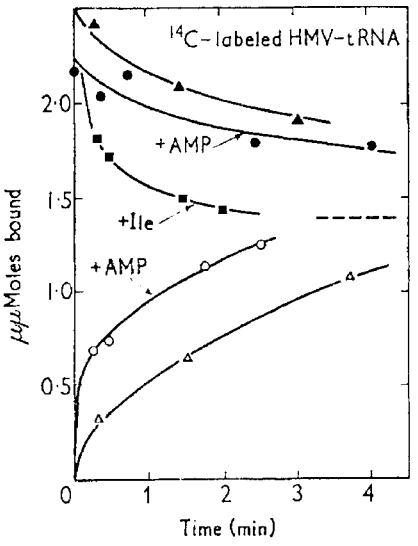


FIG. 5. Effect of AMP on the exchange of bound Ile-tRNA and free HMV-tRNA.

Reactions having a total volume of 0·40 ml., maintained at 17°C, contained 23 μμmoles of IRS, 46 μμmoles of [$^{3}$H]Ile-tRNA$^{Ile(3)}$ (for convenience only, $^{3}$H radioactivity is not plotted in Fig. 5) and 46 μμmoles of $^{14}$C-labeled HMV-tRNA$^{Ile(3)}$. 0·06-ml. samples were filtered at intervals. When present, the isoleucine concentration was $0{\cdot}5 \times 10^{-3}$ M; AMP was $1{\cdot}5 \times 10^{-3}$ M.

—△—△—, Ile-tRNA added first, no AMP; —○—○—, Ile-tRNA added first, AMP added; —■—■—, $^{14}$C-labeled HMV-tRNA added first, isoleucine added; —●—●—, $^{14}$C-labeled HMV-tRNA added first, AMP added; —▲—▲—, $^{14}$C-labeled HMV-tRNA added first, no AMP. The displacement between the last two curves is presumably an experimental error. The broken line marks the measured position of the steady state.

reaction. In the presence of Ile-tRNA and AMP, IRS(Ile-AMP) is formed at the catalytic site at the expense of the Ile-tRNA.

$$\text{Ile-tRNA} + \text{AMP} + \text{IRS} \rightleftarrows \text{IRS(Ile-AMP)} + \text{tRNA}. \qquad (2)$$

Therefore, although AMP has no intrinsic effect on tRNA release (see above and Table 3), when the tRNA bound is Ile-tRNA addition of AMP will stimulate exchange of the bound Ile-tRNA if the catalytic site is also an effector site. This effect is observed (Fig. 5) by measuring the exchange of bound Ile-tRNA with free $^{14}$C-labeled HMV-tRNA; in this case, addition of AMP increases the rate of exchange (lowermost curves). In contrast, when $^{14}$C-labeled HMV-tRNA and enzyme are combined first (two uppermost curves), AMP does not stimulate exchange: this is because AMP is unable to react with the HMV-tRNA to produce IRS(HMV-AMP) (see Materials and Methods). The middle curve (filled squares) of Figure 5 is a control that shows that slow release is not a peculiarity of $^{14}$C-labeled HMV-tRNA; when isoleucine is added to the reaction mixture, there is a stimulation in the dissociation of the IRS(HMV-tRNA). Thus, when isoleucine is inserted directly into the catalytic site, the dissociation of bound tRNA is stimulated.

Since Ile-tRNA does not stimulate its own release (Figs 1, 2 and 3), it is clear that the amino acid esterified to tRNA is no longer in effective contact with the isoleucine

binding site. This eliminates the appealing possibility that facilitated release might have evolved to speed replacement of an acylated tRNA by a new, unacylated molecule.

(c) *Effect of substrates on the equilibrium constant*

In Figure 6 are complete binding curves for enzyme and [$^{14}$C]Ile-tRNA (Fig. 6(a)) and enzyme and [$^{14}$C]tRNA$^{Ile(3)}$pCpC (Fig. 6(b)) in the presence and absence of substrates. The curves are quite similar. Addition of substrates, in each case, causes only a small decrease of the affinity of the enzyme for the tRNA. Analysis of Figure 6(b) (Yarus & Berg, 1967) suggests that the association constant of enzyme and [$^{14}$C]tRNApCpC decreases from $1\times10^{8}$ liters/mole to $5\times10^{7}$ liters/mole in the presence of ATP + isoleucine or isoleucine alone, and in Figure 6(a) we find that the affinity for Ile-tRNA goes from $1{\cdot}7\times10^{8}$ liters/mole to $1{\cdot}4\times10^{8}$ liters/mole. Because

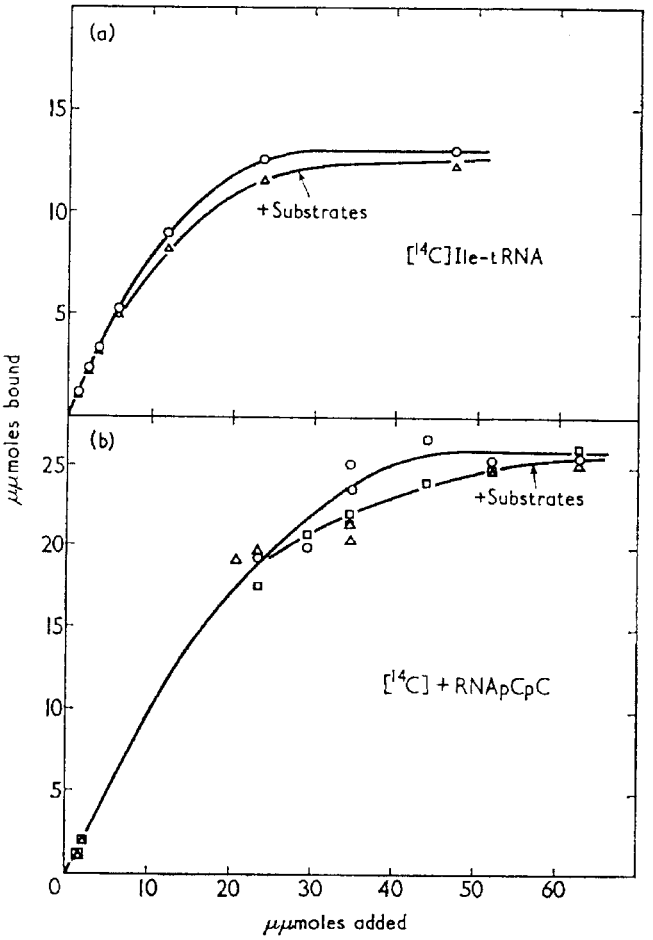


FIG. 6. A comparison of the binding of two tRNA's to IRS in the presence and absence of substrates.

(a) Effect of substrates on binding of [$^{14}$C]Ile-tRNA. tRNA is the threefold purified product of the partition chromatographic procedure of Muench & Berg (1966). IRS was converted to IRS(Ile-AMP) in the presence of $1{\cdot}0\times10^{-3}$ M-isoleucine and $0{\cdot}9\times10^{-3}$ M-ATP using the conditions of Norris & Berg (1964); 0·020 ml. of this enzyme was then used in a 0·20-ml. binding assay at 17°C (—△—△—). Alternatively, enzyme was incubated under the same conditions without substrates before use (—○—○—).

(b) Effect of substrates on the binding of [$^{14}$C]tRNApCpC. The binding assay mixtures (0·20 ml.), held at 17°C, contained either no substrates (—○—○—), $1{\cdot}0\times10^{-3}$ M-isoleucine (—□—□—), or $1{\cdot}0\times10^{-3}$ M-isoleucine plus $0{\cdot}76\times10^{-3}$ M-ATP (—△—△—).

In these Figures, the data are corrected for the efficiency of the binding assay.

this change is observed with tRNApCpC, as well as with Ile-tRNA, we conclude that occupation of the isoleucine site does not exert its effect by directly interacting with the 3′-end of bound tRNA, for example, by sterically hindering its binding; rather, the bound isoleucine probably modifies the interaction of the enzyme with the more distal part of the polynucleotide chain. In any case, the effect of substrates on the equilibrium is smaller than that on the rate of the back reaction; therefore, isoleucine must speed up both the forward and the reverse binding reactions (Table 4).

TABLE 4

*Rate and equilibrium constants for recognition of Ile-tRNA$^{Ile}$*

| Conditions | $K_e$ (l./mole) | $k_d$ ($sec^{-1}$) | $k_a$ (calc.) ($M^{-1}\ sec^{-1}$) |
|---|---|---|---|
| No substrates | $1{\cdot}7\times10^{8}$ | $7{\cdot}5\times10^{-3}$ | $1{\cdot}3\times10^{6}$ |
| ATP + isoleucine present | $1{\cdot}4\times10^{8}$ | $4{\cdot}3\times10^{-2}$ | $6{\cdot}0\times10^{6}$ |

$K_e = k_a/k_d$; $K_e$ is the equilibrium constant for association; $k_a$ is the second-order rate constant for association of IRS and Ile-tRNA and $k_d$ the first-order rate constant for the dissociation of IRS(Ile-tRNA). See Results for the origin of $k_d$ and $K_e$.

The observed strong binding of tRNApCpC and tRNApC (about $1{\cdot}2\times10^{8}$ liters/mole, calculated from competition with Ile-tRNA for IRS) suggests that the terminal cytidylate and adenylate residues contribute very slightly to the strength of interaction of tRNA$^{Ile}$ and enzyme. A systematic exposition of the effect of various 3′ modifications will be made in another communication (Yarus & Berg, manuscript in preparation).

(d) *Reciprocal effect*

Since the isoleucine site is coupled to the tRNA binding site, we may anticipate that occupation of the tRNA site will affect the properties of the isoleucine binding site in the protein. That this is the case is shown by the difference in rate of ATP–$^{32}PP_i$ exchange at different concentrations of isoleucine in the presence and absence of a saturating concentration of tRNA$^{Ile(3)}$(CHO)$_2$ (Fig. 7). The presence of tRNA

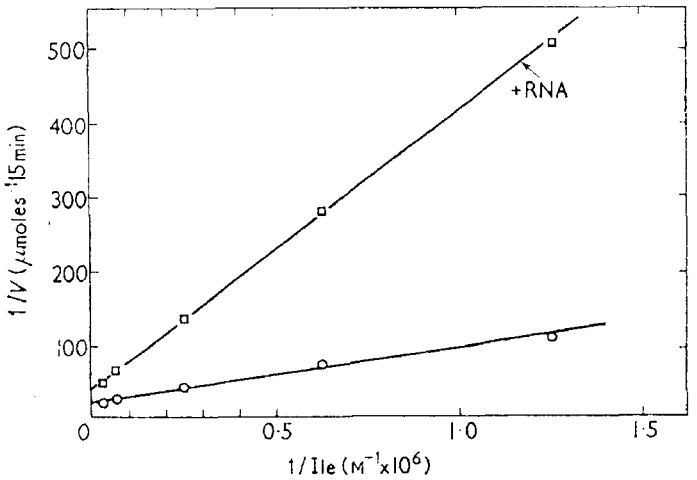


FIG. 7. Effect of tRNA on the isoleucine concentration dependence of ATP–$PP_i$ exchange. ATP–$PP_i$ exchange was conducted at 17°C under the conditions of the binding assay, with the addition of $2\times10^{-3}$ M-$^{32}PP_i$ ($1{\cdot}03\times10^{5}$ cts/min/μmole), $2\times10^{-3}$ M-ATP and 7·7 μμmoles of IRS in 0·25 ml. When tRNA was included, the equivalent of 47 μμmoles of tRNA$^{Ile(3)}$(CHO)$_2$ were added.

increases the Michaelis constant for isoleucine from $3{\cdot}6\times10^{-6}$ M to $8{\cdot}6\times10^{-6}$ M and reduces the maximum velocity from 390 moles exchanged/mole enzyme-minute to 210 moles exchanged/mole enzyme-minute. $tRNA^{Ile(3)}(CHO)_2$, then, is a non-competitive inhibitor of the exchange reaction, but the inhibited enzyme is able to carry out exchange at a reduced rate. Without further data, however, it is not possible to discern more specifically which step(s) of the exchange reaction is modified when the tRNA recognition site is occupied.

## 4. Discussion

The present studies show that isoleucyl-tRNA synthetase should be included on the list of enzymes which exhibit interactions between disparate sites (Monod, Changeux & Jacob, 1963). It is interesting in this connection that the enzyme may have little symmetry; it has only one site for Ile-AMP and, therefore, presumably only one site for ATP and isoleucine (Norris & Berg, 1964). Only one site for tRNA has been detected (Yarus & Berg, 1967).

Earlier studies have shown effects of tRNA on the amino acid-dependent ATP–$PP_i$ exchange with several aminoacyl-tRNA synthetases. Most striking are cases in which the exchange does not occur or occurs only at very high amino acid concentrations when the cognate tRNA is absent; glutamyl- and glutaminyl-tRNA synthetases from *E. coli* (Ravel, Wang, Heinemeyer & Shive, 1965), arginyl-tRNA synthetase of *E. coli* (Mitra & Mehler, 1966) and glutamyl-tRNA synthetase of rat liver (Deutscher, 1967). An explanation offered by the above authors is that binding of tRNA to the protein induces a conformational change, which either facilitates the binding of amino acid or alternatively, aminoacyl-AMP formation itself. Support for this view derives from the fact that with glutamyl-tRNA synthetase (Ravel *et al.*, 1965; Deutscher, 1967), the $K_m$ for glutamate is strikingly decreased in the presence of $tRNA^{Glu}$. The difficulty with this view is that periodate-oxidized tRNA does not promote ATP–$PP_i$ exchange with any of these systems even though (actually only established by Deutscher) the oxidized tRNA chains are also bound by the synthetase. This implies that the acceptor function of tRNA *per se* is needed to facilitate ATP–$PP_i$ exchange (cf. Fig. 7, this paper), but how or why the terminal residue of tRNA can be involved is not known. By contrast, the effect of $tRNA^{Ile}$ binding on IRS is to cause a decrease in the affinity of the enzyme for isoleucine and a reduced rate of ATP–$^{32}PP_i$ exchange; and both of these effects are independent of the intactness of the acceptor and of the $tRNA^{Ile}$. Because of these differences, the relation between the two sets of observations is unclear. Conceivably, interactions between the amino acid and tRNA sites can be manifested in a variety of ways. However, there are previous reports of phenomena which probably are related to those studied here: tRNA affects the specificity and rate of hydroxaminate formation by *E. coli* IRS (Loftfield & Eigner, 1965) and the rates of ATP–$PP_i$ exchange by rat liver alanyl-tRNA synthetase (Goldstein & Holley, 1960) and yeast IRS (Hele & Barth, 1966). These examples suggest that our observations might be characteristic of many aminoacyl-tRNA synthetases, but direct experiments are needed.

The novel finding in this study is the increase in the rate of association and dissociation of IRS and $tRNA^{Ile}$ provoked by enzymic binding of isoleucine (Figs 1 and 2; Table 1). We have measured the rate of dissociation ($k_d$) of IRS($tRNA^{Ile}$) into free IRS and $tRNA^{Ile}$ and the equilibrium constant ($K_e$) which characterizes this system: isoleucine increases $k_d$ almost sixfold and, since $K_e$ is virtually unchanged, the $k_a$ must

also increase by about sixfold (Table 4). We visualize the effect of isoleucine in the following way. It is as if occupation of the isoleucine-binding site "opens" the tRNA binding site, thereby facilitating *both* entry and exit; when isoleucine is not bound, the tRNA binding site is more "closed". Put another way, isoleucine controls the ease with which tRNA passes into and out of its binding site without altering very much the interaction of the tRNA with the groups whose disposition and character supply the strength and specificity of tRNA recognition.

The effect of isoleucine on tRNA release, as pointed out in the Introduction, must be consistent with the rate of the acylation reaction. The maximum velocity of the acylation reaction when conducted under binding conditions indicates a rate constant $5 \times 10^{-2}$ mole Ile-tRNA/mole enzyme-second (Yarus, unpublished results). This is to be compared to the maximum rate of Ile-tRNA release, $4{\cdot}2 \times 10^{-2}$ mole Ile-tRNA/mole-second (Table 4), suggesting that release of the acylated tRNA is the rate-limiting step in the aminoacylation reaction. Since under these same conditions ATP–$PP_i$ exchange can have a rate of 3·5 moles $PP_i$/mole enzyme-second (data from Fig. 7), the minimum rate of IRS(Ile-AMP) formation is 70 times faster than Ile-tRNA synthesis. Thus, the predominant state of the enzyme during the reaction, as it is usually conducted, is probably the ternary complex IRS(Ile-AMP)(Ile-tRNA).

To determine whether release of aminoacyl-tRNA is the rate-limiting step under other conditions, one could again compare the rates of release and acceptance. However, as we have pointed out (Table 1, legend), the maximum rate of release at 17°C is already at the limit of measurability by manual techniques. Therefore, pending completion of a fast sampling device, we have characterized the reaction by measuring its activation energy. Figure 8 is an Arrhenius plot of data collected under conditions usually employed to measure aminoacyl acceptance (cacodylate buffer, pH 7, squares), or under the conditions of the binding assay (potassium phosphate buffer, pH 5·5,

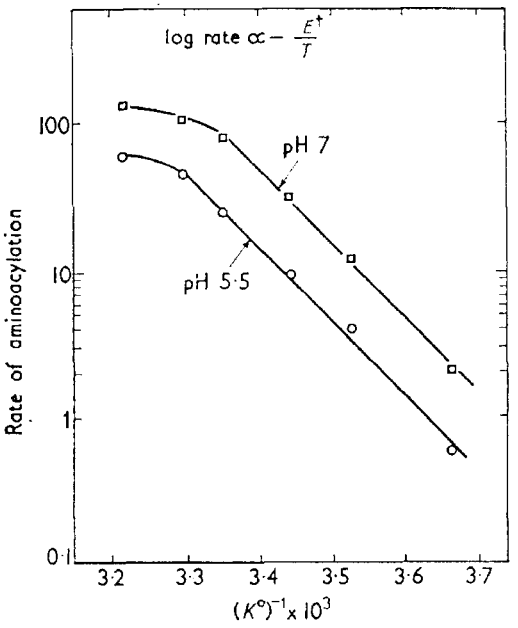


FIG. 8. Activation energy ($E\dagger$) of the aminoacylation reaction.

The rate, plotted in arbitrary units, was determined under the usual conditions (Berg *et al.*, 1961) (—□—□—), and also in 0·20 ml. binding assay solution (—○—○—) with the addition of $1{\cdot}02 \times 10^{-5}$ M-[$^{14}$C]isoleucine, $2{\cdot}0 \times 10^{-3}$ M-ATP, and 11 $A_{260}$ units of tRNA, containing 0·42 $\mu\mu$mole $tRNA^{Ile}$.

circles), both at temperatures from 0 to 37°C. The reaction is about threefold faster at pH 7, but aminoacyl transfer has the same activation energy (24 $\pm$ 2 kcal./mole) over the range 0 to 25 or 30°C, under both conditions. The rate-limiting process is probably the same, therefore, in both solvents over this temperature range. The change in activation energy above 30°C does not necessarily indicate a new rate-limiting process, and direct measurement will be necessary in this region. We take this to indicate that the action of IRS can be limited by the rate of aminoacyl-tRNA release when the reaction occurs under the usual aminoacylation conditions as well as under the conditions used for the measurement of binding.

If release of aminoacyl-tRNA is generally the rate-limiting step in aminoacylation of tRNA's, then the work of Loftfield & Eigner (1967,1968) on the velocity of acylation would appear in a new light. In particular their (1967) finding that increasing ionic strength increases the apparent $K_m$ of $tRNA^{Val}$ (*E. coli*) without affecting the maximum velocity could imply that ionic strength affects only the rate constant for association of enzyme and $tRNA^{Val}$; conceivably, the configuration assumed by $tRNA^{Val}$ at ionic strengths above 0·1 is less rapidly adapted to the enzyme surface. In addition, if acylation is not rate limiting, their (1968) suggestion that acylation is subject to general base catalysis, made to explain the stimulatory effect of $NH_3$, Tris and imidazole on the reaction, might have to be revised, since effects on the over-all velocity of the reaction could not be assumed to reflect the velocity of aminoacylation.

The increased rate of dissociation of aminoacyl-tRNA synthetase and its cognate tRNA in the presence of substrates might also explain the puzzling conclusion of Lagerkvist, Rymo & Waldenstrom (1966), who suggested that the apparent affinity of Val-tRNA synthetase for $tRNA^{Val}$ was weakened in the presence of its other substrates, ATP and valine. They isolated the complex by gel filtration. Since the Val-tRNA synthetase (Val-tRNA)(Val-AMP) would separate from free Val-tRNA as it is filtered through the gel, any increase in the rate of release of Val-tRNA caused by the Val-AMP would result in a lower recovery of the synthetase (Val-tRNA) complex.

The present findings bear on our previously described (Yarus & Berg, 1967) assay using competition as a means of detecting tRNA's capable of being bound by IRS. For the assay measuring competition between two different tRNA's to be valid, the system must be at equilibrium; otherwise, if the amount of tRNA complexed to the protein is measured too quickly, the ratio of competing tRNA's bound to enzyme may reflect their relative rates of association, and not necessarily their relative affinities for enzyme. So that equilibrium can be reached within a short time, isoleucine must be present. Indeed, competition in the presence and absence of isoleucine should be useful in separating effects on the rate of association and equilibrium constant of various tRNA derivatives.

The rate constant for association of $tRNA^{Ile}$ and IRS (see Table 4) is two orders of magnitude less than the rate constant would be if the reaction were diffusion controlled ($4 \times 10^8$ $M^{-1}$ $sec^{-1}$)†. Obviously, the rate at which the protein and tRNA collide does

† Calculated from the equation

$$k = \frac{2\pi N}{1000} (D_{tRNA} + D_{enz}) (r)$$

(Alberty & Hammes, 1958), in which $k$ is the diffusion-controlled rate constant, N is Avagadro's number, the $D$'s are the respective diffusion constants and $r$ is the reaction radius, or radius of the recognition site. $D_{tRNA}$ is $7{\cdot}8 \times 10^{-7}$ $cm^2$ $sec^{-1}$ (Tissières, 1959); $D_{enz}$ is $4{\cdot}3 \times 10^{-7}$ $cm^2$ $sec^{-1}$ (Baldwin & Berg, 1966), and $r$ is taken to be $10^{-7}$ cm.

not limit the rate of association. Considering the complexity of the interaction between protein and nucleic acid, it is perhaps surprising that the observed rate constant ($6\times10^6$ M$^{-1}$ sec$^{-1}$, 17°C) is so large; as large, in fact, as the constants for association of some enzymes with small molecule substrates (cf. Eigen & Hammes, 1963). A reaction between macromolecules, like this one, may involve extensive, and therefore rather slow, conformational changes in both reactants, For example, conformational changes in proteins have rates of about $10^{-1}$ to $10^{-4}$ sec$^{-1}$ (Berger, Antonini, Brunori, Wyman & Rossi-Fanelli, 1967; Kirschner, Eigen, Bittman & Voight, 1968; Havsteen, 1967; McConn, Czerlinski & Hess, 1968), and the unwinding of tRNA helices requires several hundred microseconds and may, therefore, be assigned a rate of $10^{-3}$ to $10^{-4}$ sec$^{-1}$ (Scheffler & Baldwin, personal communication). Can such slower processes be a part of tRNA binding which proceeds at $6\times10^6$ M$^{-1}$ sec$^{-1}$? More precisely, can a conformational transition be required for formation of the strongly bound, specific IRS(tRNA$^{\text{Ile}}$) detected by the filter assay? To discuss this question, we divide tRNA binding into two steps about which we have independent information.

$$\text{Ile-tRNA} + \text{IRS} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{IRS(Ile-tRNA)}]^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{IRS(Ile-tRNA)}.$$

The first step may be visualized as analogous to the binding of ordinary small molecules by enzymes. We suppose that it leads to a tentative association of enzyme and tRNA [IRS(Ile-tRNA)]* which is easily disrupted and, therefore, not detected after filtration. There follows the hypothetical conformational transition involving large readjustments of the enzyme, tRNA, or both to form the strongly bound IRS(Ile-tRNA). This second form is detected on filters. We have, using data from Table 4:

$$K_e = \frac{k_a}{k_d} = \frac{k_1 k_3}{k_4} = \frac{6\times10^6}{4{\cdot}3\times10^{-2}} \cong 10^8 \text{ liters/mole}.$$

Thus, if $k_3$ were as slow as $10^{-1}$ to $10^4$ sec$^{-1}$ (see above), $k_1$ could range from $6\times10^7$ to $6\times10^2$ and still be consistent with the measured parameters for $k_d$ and $K_e$. These values of $k_1$ overlap the range observed for small molecule–enzyme associations, as summarized by Eigen & Hammes (1963). Therefore, our data will accommodate rather slow, known types of conformational transitions, if the formation of a transient intermediate precedes the change in macromolecular structure. Our data do in fact suggest that some sort of conformational change probably occurs during this interaction. The conformational change alters the $K_m$ for isoleucine binding and the $V_{max}$ of the ATP–$^{32}$PP$_i$ exchange reaction (Fig. 7). This fact is, therefore, not in conflict with a high rate of interaction ($k_a$), even if this latter conformational change is required for effective tRNA binding.

We have attempted to summarize our findings in Figure 9, which is a diagram of the active cycle of IRS. For purposes of simplicity and clarity, the reversibility of the reactions shown and conceivable side reactions have been ignored. A key part of the illustration is the difference in the state of the tRNA binding site when isoleucine is bound and in the isoleucine site when tRNA is bound. Steps 1 and 2 show the binding and reaction of ATP and isoleucine to form IRS(Ile-AMP) with the elimination of PP$_i$. At step 3, the incoming tRNA is bound to the site "opened" by occupation of isoleucine in step 1; in addition, the binding of tRNA influences the isoleucine site. In step 4, the ternary complex IRS(Ile-AMP)(tRNA$^{\text{Ile}}$), formed in reaction 3, undergoes the transacylation reaction producing IRS(AMP)(Ile-tRNA$^{\text{Ile}}$). Although we

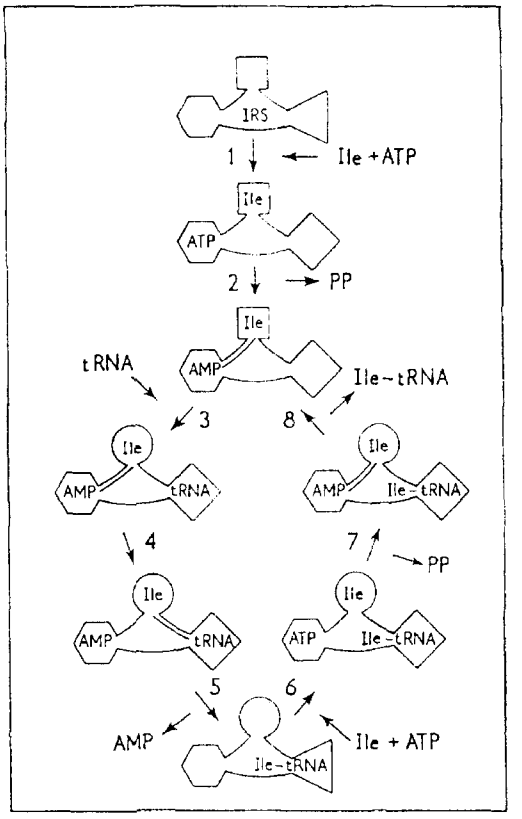


FIG. 9. A possible catalytic cycle for IRS.

have no direct information on this point, it is conceivable that dissociation of AMP (step 5) causes the isoleucine moiety of the Ile-tRNA to be ejected from the isoleucine binding site. We know, on one hand, that the isoleucine in Ile-tRNA does not maximally stimulate its own release from the enzyme, and, therefore, is probably not in effective contact with the isoleucine site. It is, therefore, shown to leave this site in step 5 of Figure 9. On the other hand, we know that isoleucine can re-enter this site because when AMP is added to IRS(Ile-tRNA$^{Ile}$), the unbound isoleucine portion of the Ile-tRNA must be inserted into the catalytic site to form Ile-AMP. We therefore suggest that, as shown in step 5, AMP may have a role in determining the disposition of the isoleucine of Ile-tRNA.

We assume that there is very rapid addition of ATP and isoleucine to the vacant sites on the IRS(Ile-tRNA$^{Ile}$) (in step 6) and formation of the Ile-AMP derivative (step 7). This facilitates the rate-limiting step, the dissociation of the bound Ile-tRNA (step 8), permitting another catalytic cycle after a new molecule of tRNA$^{Ile}$ is bound.

Figure 9 portrays the enzyme as existing in four discrete conformational states, but our data, taken at face value, only *require* two states: for example, one which releases tRNA quickly, and another characterized by slower tRNA release. Detailed consideration of our data suggests, however, that two states are quantitatively inconsistent with our observations, and that IRS must have at least three conformational states. Thus, there is still insufficient experimental information to characterize fully the scope of the conformational changes the enzyme undergoes during a round of catalysis, but we have chosen the four-state model for discussion because it incorporates the

intuitively appealing idea that the binding of each ligand provokes its own characteristic conformational change.

Perhaps some comment is in order regarding the broader significance of an interaction between amino acid and tRNA binding sites. Because the affinity of the enzyme is changed only slightly by substrates (Fig. 6), the level of IRS($tRNA^{Ile}$) will not be affected by amino acid†; therefore, we think it unlikely that the isoleucine-induced molecular transition is part of a regulatory mechanism dependent on the concentration of IRS($tRNA^{Ile}$). Rather, we think it likely that the binding assay has disclosed a regular intermediate state of the acylation reaction; that is, we presume that a mobile recognition site is required for enzyme function, and one consequence of its required polymorphous nature is the difference in binding rates.

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† At least 200 molecules of IRS and 1100 molecules of $tRNA^{Ile}$ may be isolated from each *E. coli* B cell (calculated from the yield of purification procedures). Therefore, in a cell volume of $6\times10^{-16}$ liter, the concentrations of synthetase and $tRNA^{Ile}$ must be at least $5\times10^{-7}$ and $3\times10^{-6}$ M. A change of about two orders of magnitude in the dissociation constant ($10^{-8}$ mole/liter) of IRS($tRNA^{Ile}$) would be required to effect large enough changes in the amount of complex to regulate any process which depends on the concentration of IRS($tRNA^{Ile}$).

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